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Biodegradation of Polyethylene by *Bacillus* sp. Indigenous to the Niger Delta Mangrove Swamp

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Abstract

The ability of *Bacillus mycoides and Bacillus subtilis* (*Bacillus* species indigenous to the Niger Delta mangrove soil) to biodegrade polyethylene was studied. Low-density polyethylene (LDPE) and high-density polyethylene (HDPE) films were exposed outdoor for 24 weeks. The two isolates were able to grow on polyethylene (PE), forming visible biofilms. The plasmid pattern of the *Bacillus* species showed a similar pattern among the two *Bacillus* species, with one plasmid number and molecular weight around 25 kb, indicating a mega plasmid. The mean heterotrophic bacterial counts in the mangrove soil ranged between 2.81×10^5 – 3.20×10^8 CFU/g. The rate of degradation was determined by measurement of the residual weight of the PE films. Biodegradation in Erlenmeyer flasks by the bacteria after 60 days of incubation ranged between 8.41%-23.15%. Biodegradation was confirmed by Fourier-Transform Infrared (FTIR) spectroscopy, which showed introduction of carbonyl groups after natural weathering, which decreased after microbial treatment. Decrease in carbonyl index ranged between 10.5%-13.7%. The result showed that certain *Bacillus* sp. indigenous to the Niger Delta mangrove soil are capable of growing on PE films and biodegrade them, after an initial abiotic degradation.

Key words: *Bacillus mycoides*, *Bacillus subtilis*, biodegradation, carbonyl index, Natural weathering, Niger Delta mangrove.

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Introduction

Polyethylene (PE) littering of the environment is a common problem in most urban centers in Africa, because majority of PE wastes are often not recycled in most countries in Africa. This is because of the poor waste management practice in Africa. Within the last few decades there has been an increasing rise in plastic waste entering into the municipal solid waste streams in large cities in sub-Sahara Africa. These plastic wastes are causing an increasing number of environmental and health problems to humans and other animals. It has reportedly caused over a million deaths of marine animals (Barnes *et al*., 2009). These wastes have virtually choked the drainage system in the urban centers of countries in this region, to such an extent that it takes only the slightest of rainfall to precipitate floods in major cities in these areas, as is presently witnessed in Yenagoa.

Almost all the plastics entering the environment in sub-Sahara Africa are either burnt, buried or left to degrade naturally. Degradation of polymers in nature is well documented. It follows a sequence in which the polymer is first converted to its monomers, before they are mineralized. Most polymers like PE are too large to pass through cellular membranes, so they must first be depolymerized to small monomers before they can be absorbed into the microbial cells. The degradation of most synthetic plastics in nature is a very slow process taking over a thousand years and involves the synergistic action of environmental factors and microorganisms (Cruz-Pinto *et al*., 1994; Nanda & Sahu, 2010). Some studies (Glass & Swift, 1989; Imam *et al*., 1992; Gu, 2003) have assessed the biodegradability of some PE films by measuring changes in physical properties, amount of CO_2 evolved or by observation of microbial growth after exposure to biological or enzymatic environments. Gilan *et al*. (2004) and Hadad *et*

al. (2005) reported that abiotic pretreatment of polyethylene leads to formation of carbonyl compounds upon which certain microorganism can act.

Recent researches have revealed that the high diversity of the microorganisms in the mangrove soil which is rich in nutrients and organic matter includes bacteria capable of degrading plastics, although at a slower rate (Katherisan, 2003; Kumar *et al*., 2007). Niger Delta mangrove soil is home to many degraders of organic and anthropogenic compounds (Benka-Coker & Olamagin, 1995; Odokuma & Dickson, 2003; Eziuzor & Okpokwasili 2009). There are several published works on biodegradation of hydrocarbon by mangrove soil microbial isolates from the Niger Delta but information on their ability to biodegrade plastics is lacking. Sahoo & Dhal (2009) reported that there are potential microbes in the mangrove ecosystem on which much study have not been done yet. An attempt has been made in this paper to study the biodegradation of naturally weathered HDPE and LDPE films by Niger Delta mangrove soil bacterial isolates. The extent of biodegradation was evaluated by comparing the initial and final dry weights of the PE, before and after incubation. FTIR spectroscopy evidence was further used to confirm biodegradation of the PE films.

Materials and Methods

Sample collection and source of Polyethylene: The soil was collected from Koluama mangrove located in Southern Ijaw LGA, an oil producing community of Bayelsa State in the Niger Delta region of Nigeria. The sampling area lies within latitude 4^o 27′ N and 4^o 30′ N and longitude 5^o 47′ E and 5^o 50′ E. The sample was excavated with a trowel at 5cm depth and collected in a plastic crates during low tide. The polyethylene films, High density polyethylene (45 µm) and low density polyethylene (42 µm) were supplied by Bayelsa State Plastics Company.

Preliminary weathering of polyethylene: Pre-weighed LDPE and HDPE films were exposed to sunlight on the roof of a 4.5m tall building for 6 months, between January and June. Exposure was performed according to ASTM standards, using 45°-angle wooden racks, facing south (Yabannavar & Bartha, 1994).

Isolation, enumeration and identification of polyethylene degrading fungi: Isolation and enumeration: Soil samples were collected from the upper 0-5 cm layer of the mangrove soil, stored in plastic crates and transported to the Environmental Microbiology laboratory of University of Port Harcourt, where it was kept at room temperature. The cut PE films were then buried in the soil after liming with Ca_2CO_3 and fertilizer [$(NH_4)_2HPO_4$] application. After 1,2 and 3 months of burial, the PE pieces were removed nd transferred onto nutrient agar (NA) plates and incubated at 30^oC for 24 hours, for bacterial isolation from the surface of PE. The direct isolation process was carried out by adding 1 g of soil to 9 ml of sterile distilled water in a test tube to yield a 10-fold dilution. Next, a series of 10-fold dilutions were made in which 1 ml of each dilution was cultured on NA. Pure cultures were finally obtained by selecting a single colony of growth from highly diluted cultures. After incubation, the plates showing the most growth were chosen for enumeration.

Identification of polyethylene degrading bacteria: The identification of the bacterial isolates with the ability to degrade PE was performed on the basis of macroscopic and microscopy examination and biochemical tests. The bacterial isolates were identified macroscopically by examining colony morphology; surface pigment, size, margin and microscopic examination including Gram staining and motility test. The biochemical tests conducted were sugar fermentation, nitrate reduction, oxidase, citrate and catalase tests. The bacterial isolates were identified based on the keys detailed by Aneja (2003). Further identification was carried out using API 50 CHB analytical kit.

Isolation of plasmid DNA: Isolation of plasmid DNA was carried out by boiling. After electrophoresis in 0.8% Agarose gel, the gel was stained with 0.5 µg/ml ethidium bromide. The marker used is Hind III digested Lambda phage DNA (2.027-23.130 kilobases).

Biodegradation of polyethylene: One ml culture suspension of isolates was added to Erlenmeyer flasks containing 150 ml of the mineral salt medium which contained polyethylene as the sole carbon source and incubated at 30 ± 2^oC. The composition of the mineral salt medium is as follows: (g/l: 1.0 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.0 g K_2HPO_4, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.15 g KCl, and 1.0 mg of each of the following microelements: $FeSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4$).

Fourier transform infrared spectroscopy (FTIR) was used to confirm biodegradation by determining the formation of new functional groups or disappearance of groups in the polymer ((Milstein *et al*., 1994). Changes in the polyethylene structure following natural weathering and subsequent incubation with the fungal isolates were analyzed by FTIR spectroscopy (Perkin Elmer Spectrum BX11). The carbonyl index was measured from the FTIR spectrum in the transmittance mode, by comparing the relative intensities of the carbonyl band at approximately 1715 $cm^{-}1$to that of the methylene band at approximately 1465 $cm^{-}1$.

Results

Isolation and identification of microorganisms capable of degrading polyethylene: Table 1 shows the mean heterotrophic bacterial load in the mangrove soil. The mean heterotrophic bacterial count ranged from 2.81×10^5 - 3.20×10^8. The microbial count increased steadily with increase in time of burial. There were more microorganisms attached to LDPE films than to HDPE. Among the isolates screened for ability to degrade the LDPE and HDPE films, two bacterial isolates exhibited the fastest growth. The isolates were identified as *Bacillus mycoides* and *B. subtilis*.

Table 1: The mean heterotrophic bacterial count in the mangrove soil from Koluama mangrove, Nigeria

EXPOSURE TIME	HDPE	LDPE
0	2.81×10^5	2.81×10^5
1	2.90×10^6	3.38×10^6
2	1.41×10^7	2.35×10^7
3	2.46×10^8	3.20×10^8

Table 2: Morphological and biochemical characteristics of *Bacillus mycoides* and *Bacillus subtilis*

Charateristics	Bacillus mycoides	Bacillus subtilis
Morphology		
Straight rod	+	+
Gram's Reaction	+	+
Cell arrangement	Short chain, single	Short chain, single
Spore	Central	Central
Motility	-	+
Sugar fermentation		
Glucose	+	+
Lactose	-	+
Sucrose	+	+
Fructose	+	+
Mannose	+	+
Sorbitol	-	+
Oxidase	-	+
Catalase	+	+
Citrate	+	+
Nitrate reduction	+	+

+, Positive; -, Negative

Plasmid Patterns: The plasmid pattern of the *Bacillus* species is presented in Figure 1. Results showed the same plasmids pattern among the two *Bacillus* species. The plasmid number is one and the molecular weight of the plasmid is around 25 kb, indicating a mega plasmid.

Marker *Bacillus mycoides* *Bacillus subtilis* Marker

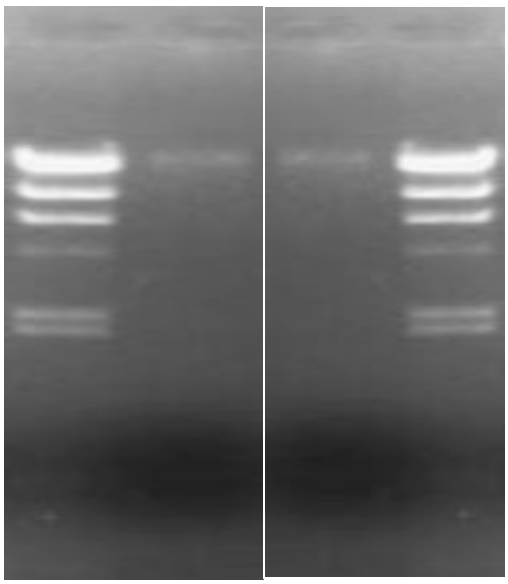


Figure 1: Plasmid profile of *Bacillus* species isolated from mangrove soil subjected to electrophoresis on 0.8% Agarose gel. The marker used is Hind III digested Lambda phage DNA.

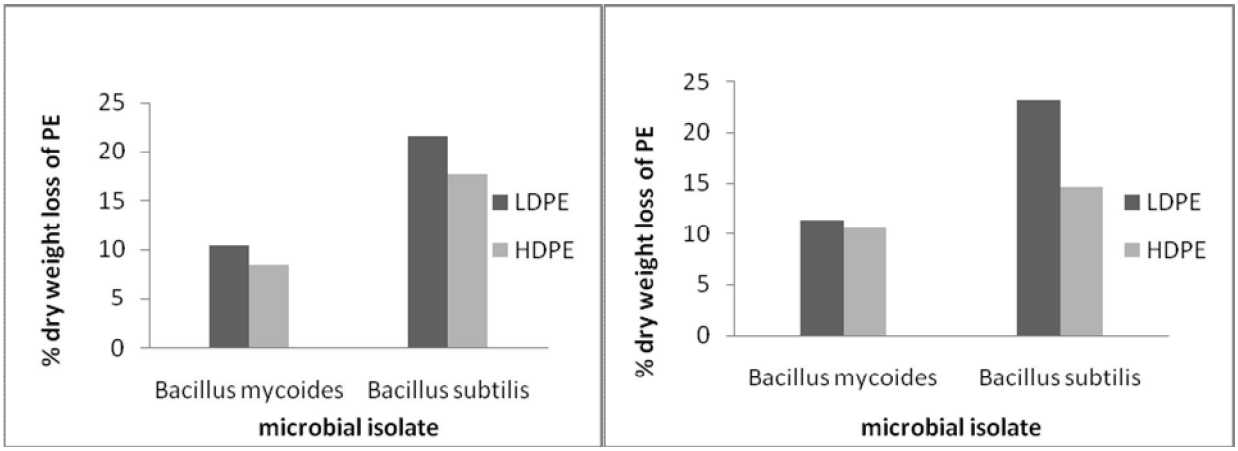
Residual weight measurement for films incubated with microbial isolates: The results of residual weight measurement of pre-exposed and unexposed LDPE and HDPE applied to microbial treatment by incubating with individual isolates and microbial consortium in mineral salt liquid medium, expressed as percentage loss in weight, are given in Table 3 & 4. Figure 2 showed that both isolates degrade LDPE better than HDPE and that biodegradation rate by *Bacillus subtilis* exceeds that of *Bacillus mycoides*.

Table 3: percentage biodegradation of PE films by isolates after 45 days of incubation at room temperature

TREATMENT	PERCENTAGE WEIGHT LOSS (HDPE)	PERCENTAGE WEIGHT LOSS (LDPE)
Pre-exposed film + *Bacillus mycoides*	8.41 ± 0.14	10.40 ± 0.35
Pre-exposed film + *Bacillus subtilis*	15.50 ± 0.38	21.60 ± 0.36

Table 4: Percentage biodegradation of PE films by isolates after 60 days incubation at room temperature

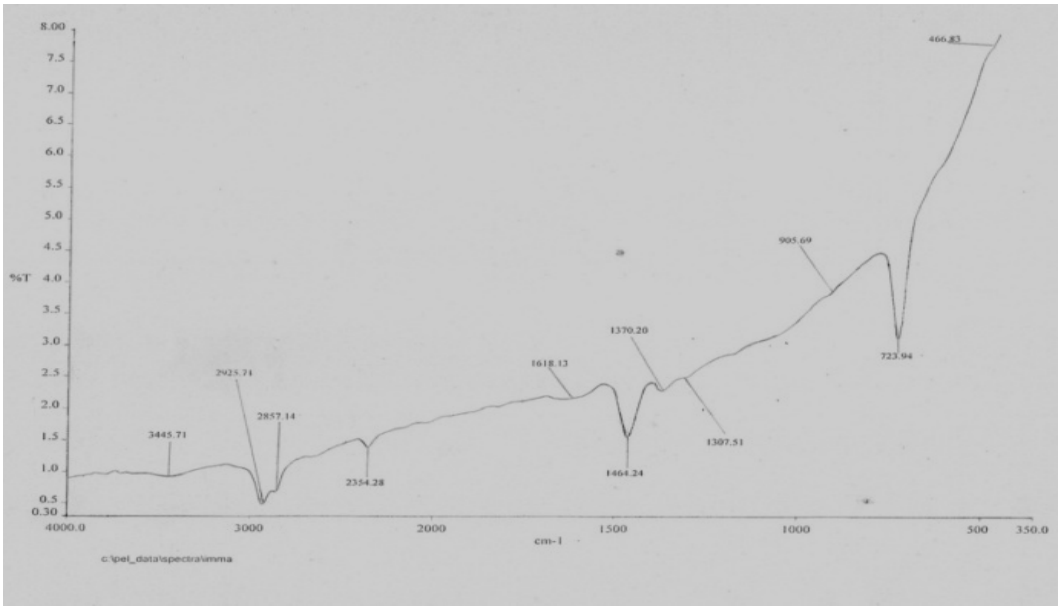
TREATMENT	% WEIGHT LOSS (HDPE)	% WEIGHT LOSS (LDPE)
Pre-exposed film + *Bacillus mycoides*	10.50 ± 0.17	11.35 ± 0.05
Pre-exposed film + *Bacillus subtilis*	17.72 ± 0.10	23.15 ± 0.05



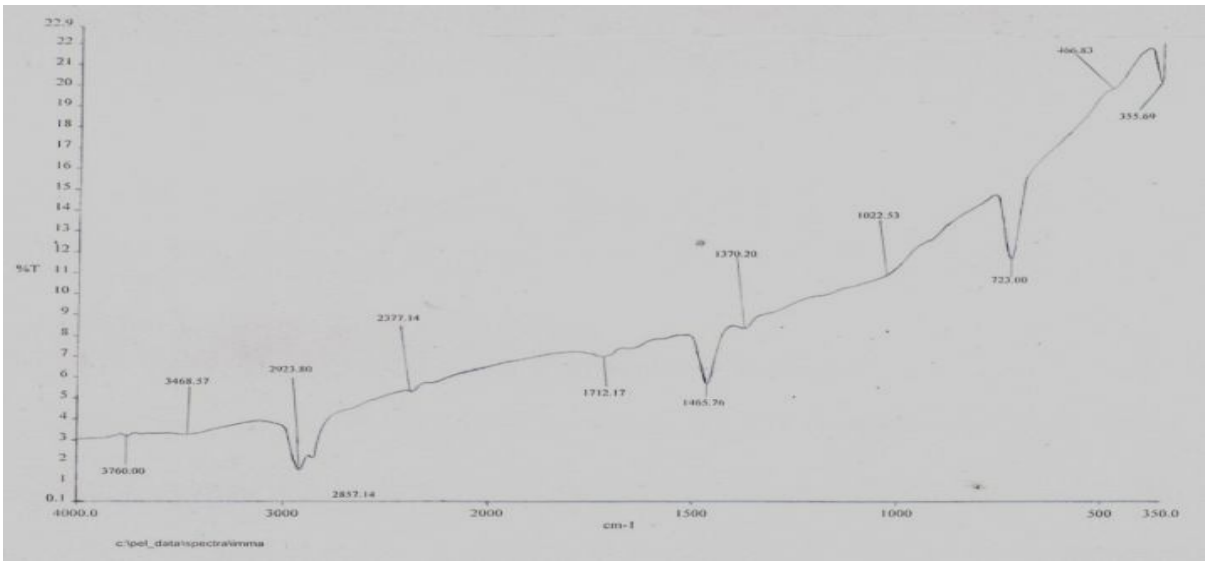
(a) Percentage dry weight after 45 days of incubation (b) Percentage dry weight after 60 days of incubation

Fig. 2: percentage dry weight of LDPE and HDPE after microbial treatment

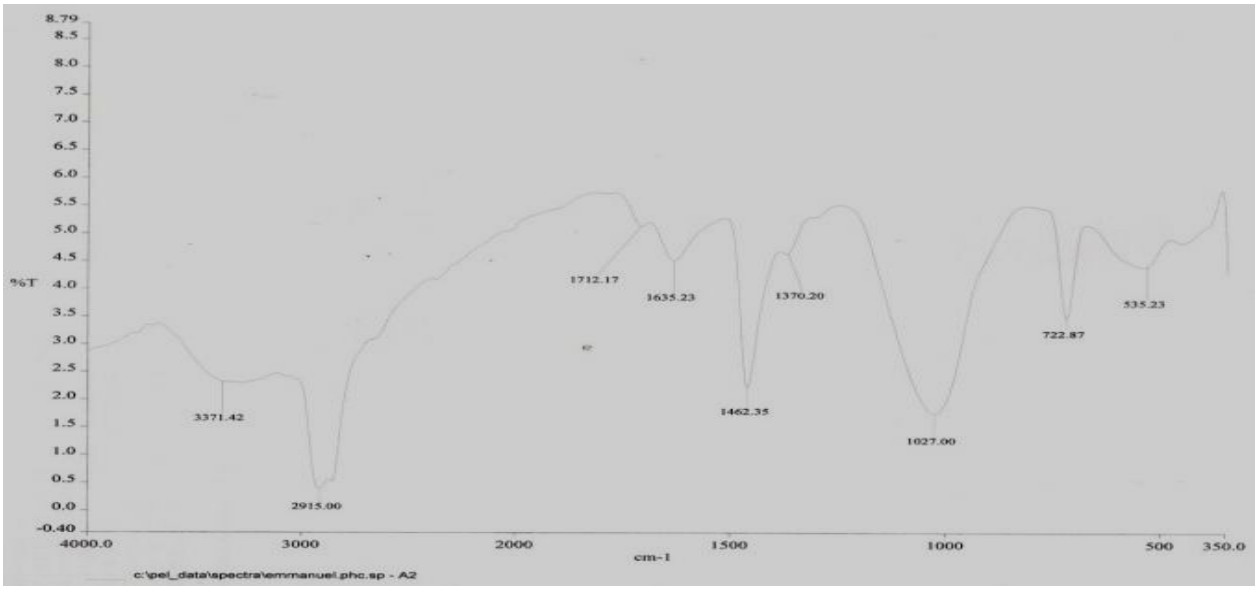
FTIR spectra result for LDPE and HDPE: Biodegradation of PE films was confirmed using FTIR spectroscopy and the result is given in Figure 3. Figure 3 shows FTIR spectra for virgin PE, naturally weathered PE and films incubated in Erlenmeyer flasks with microbial isolates from mangrove soil in which the films served as the sole carbon source in a carbon-free mineral salt medium. Table 5 and 6 showed the carbonyl index for LDPE and HDPE respectively. Fig. 3A is spectra pattern for virgin PE film which does not show presence of carbonyl groups. Fig. 3B shows introduction of carbonyl group into the polymer matrix as a result of abiotic oxidation. Absorbance around 1712 cm^{-1} and 3100 - 3700 cm^{-1} indicate presence of oxygen bounded compounds in the treated films which are sites for microbial action. Fig. 3C-H show reduction and disappearance of some chemical functionalities introduced into the abiotically treated films as a result of microbial oxidation of these compounds. Tables 5 & 6 show the carbonyl index of the films, showing increase after exposure to sunlight and decrease when the films were applied to microbial treatments.



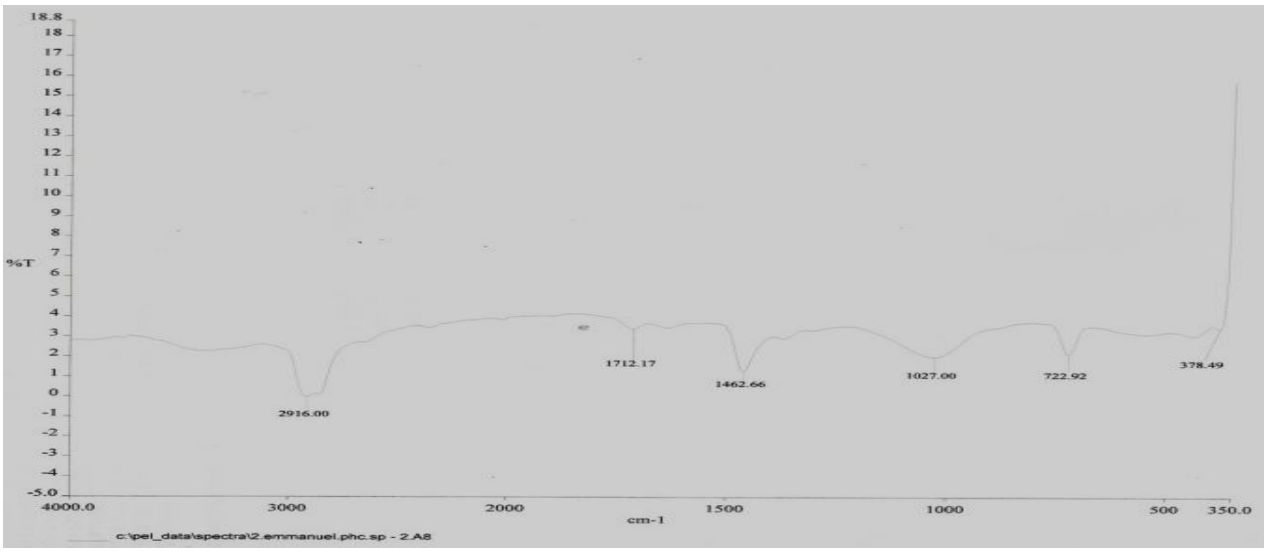
(A) Virgin LDPE film



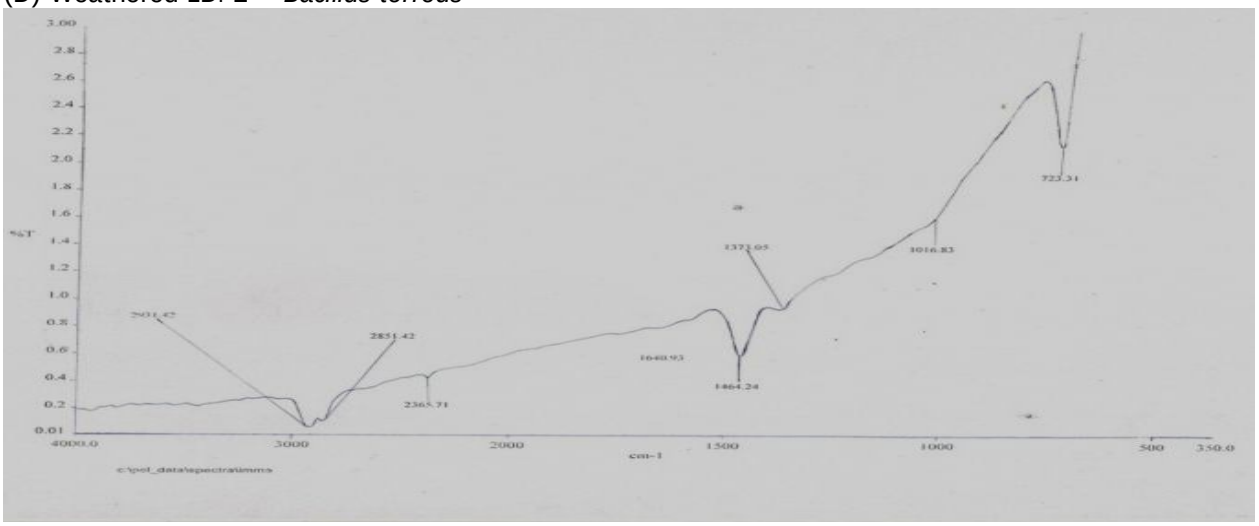
(B) Naturally weathered LDPE



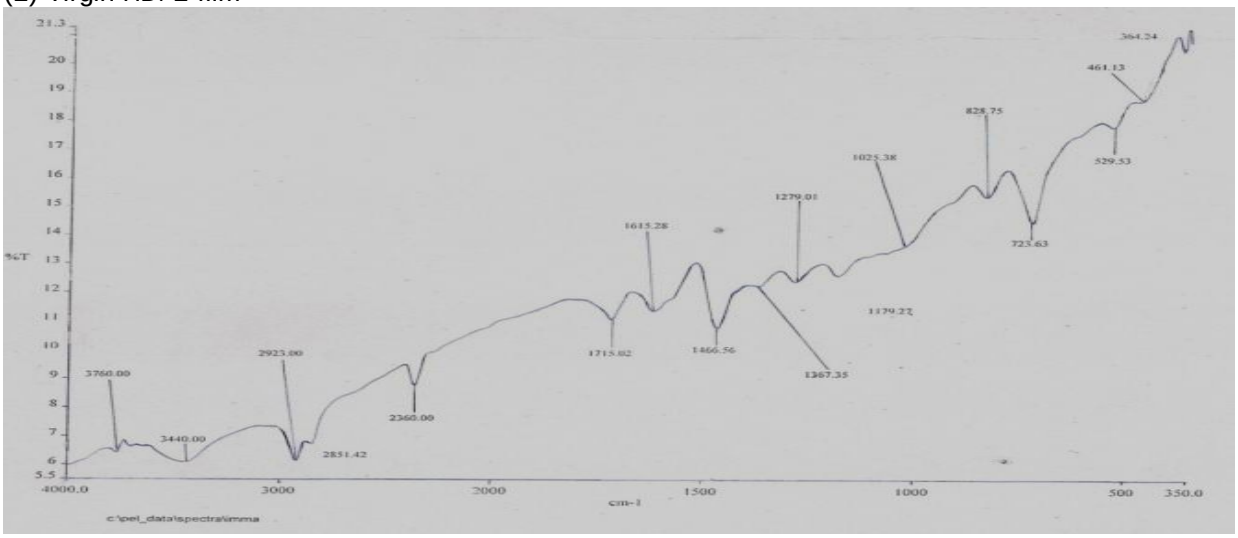
(C) Weathered LDPE + *Bacillus mycoides*



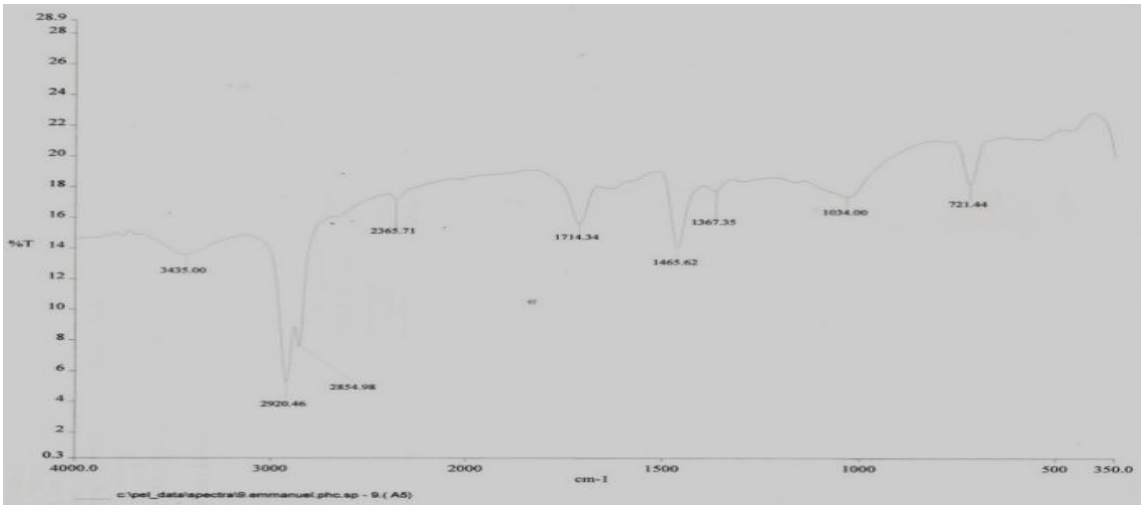
(D) Weathered LDPE + *Bacillus terreus*



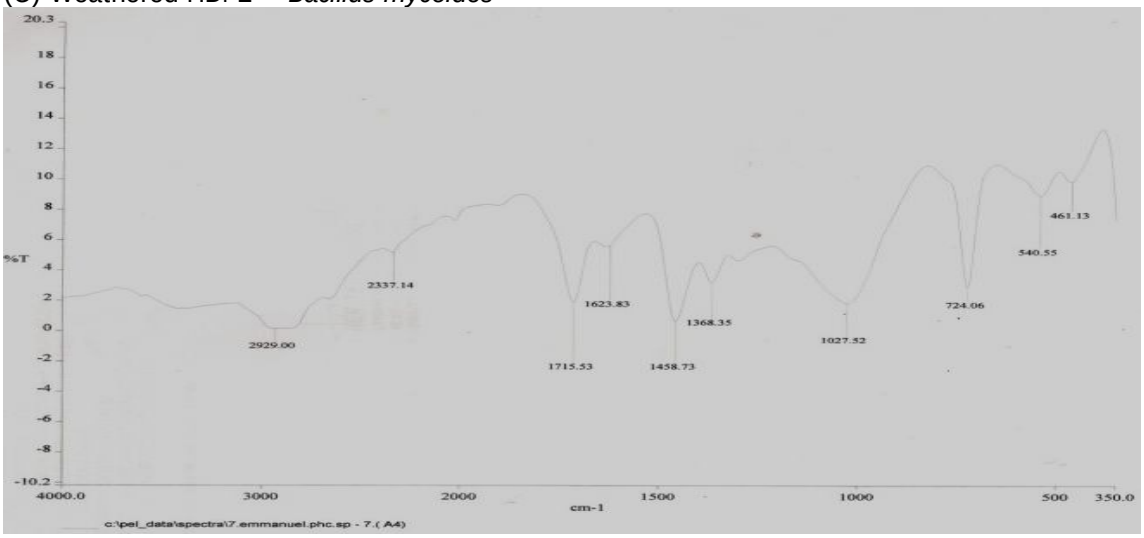
(E) Virgin HDPE film



(F) Naturally weathered HDPE



(G) Weathered HDPE + *Bacillus mycoides*



(H) Weathered HDPE + *Bacillus subtilis*

Figure 3: FTIR spectra of pre-treated and treated LDPE and HDPE films

Table 5: Carbonyl index obtained from FTIR spectra of LDPE incubated with microbial isolates for 60 days at room temperature

Treatment	Carbonyl index [C=O : CH_2]
Non-treated (control)	1.29 ± 0.07
Naturally weathered	1.61 ± 0.08
Naturally weathered + *Bacillus mycoides*	1.39 ± 0.03
Naturally weathered + *Bacillus subtilis*	1.18 ± 0.03

Table 6: Carbonyl index obtained from FTIR spectra of HDPE incubated with microbial isolates for 60 days at room temperature

Treatment	Carbonyl index [C=O : CH_2]
Non-treated (control)	1.01 ± 0.03
Naturally weathered	1.52 ± 0.10
Naturally weathered + *Bacillus mycoides*	1.36 ± 0.01
Naturally weathered + *Bacillus subtilis*	1.20 ± 0.04

The carbonyl index expresses the ratio between the absorbance for keto carbonyl group at approximately (1715 cm^{-1}) and that of the CH_2 group at approximately 1465 cm^{-1}. Values are means ± S.D. (n=3).

Discussion

The results revealed that the heterotrophic bacteria count ranged from 2.81 x 10^5 – 3.20 x 10^8 CFU/g. The total heterotrophic bacteria (THB) count was found to agree with the THB from Oron mangrove sediment in Nigeria (Ekpo & Madu, 2005). The high count of the heterotrophic bacteria in the mangrove soil is considered advantageous because they contribute significantly to the microbial decomposition process in the soil. Results indicated an increase in count after three months of burial. Orhan *et al*. (2004) reported that an increase of bacterial population correlated with the signs of disintegration of mechanical properties of natural polymer films, indicating the role of biotic component in degradation process This result is similar to the observations of Kathiresan, (2003) who investigated the degradation of plastics in mangrove soil and observed that significant biodegradation occurred only after colonization of the plastic, a parameter that was dependent on the resident microbial populations.

Bacillus sp have been isolated from the soil in the Niger Delta with some associated with the degradation of hydrocarbons associated with crude oil (Antai, 1990; Akpan-Idiok & Solomon, 2012; Eziuzor & Okpokwasili 2009). There is well documented evidence of indigenous Niger Delta soil microbes degrading the oil pollutants entering the terrestrial and aquatic ecosystem (Benka-Coker & Olamagin, 1995; Odokuma & Dickson 2003). The same microorganisms that mediate the degradation of hydrocarbon are expected to degrade polyethylene since their degradation is similar (Arkatkar *et al*., 2009). Both *Bacillus mycoides* and *Bacillus subtilis* exhibited varying degrees of ability to biodegrade PE and it was assumed that their isolation from soil constantly polluted by oil spill could confer such degradative ability on them.

Biodegradation in Erlenmeyer's flask showed no growth of the bacterial isolates on the untreated films. However, growth was observed on treated films serving as sole carbon sources, resulting in weight loss of the films. Result of residual weight measurement revealed a dry weight loss ranging from 10.4% - and 23.15 % for LDPE and 8.41% - 17.72% for HDPE at the end of the incubation period for treated films probably because of the presence of carbonyl groups while the untreated films remained unchanged. A similar result was obtained by Sudhakar *et al*. (2007) when *Bacillus* species isolated from the marine environment was used to degrade thermally treated LDPE and HDPE (19% and 9% respectively). 2004 reported 11% reduction in gravimetric weight of polyethylene after treatment with *Brevibacillus borstelensis* and Kathiresan (2003) reported 20.54% by *Pseudomonas* species. These low rates of degradation are in agreement with the argument of Otake *et al*. (1995) that 10 years is a relatively short period for the biodegradation of synthetic polymers such as polyethylene.

It has been severally reported that bacterial flora growing in a stressed environment usually harbor different types and numbers of plasmid (Baya *et al*., 1986). Plasmid profile of the isolates exhibited a single band with the size of around 25 kb in both isolates. Kamala-Kannan and Lee (2008) have reported the presence of large plasmids in *Bacillus* species, similarly in the present study, a large plasmid was observed in the two *Bacillus* species. Plasmids play important roles in *Bacillus* sp. Large

variety of specific biochemical functions such as fertility, resistance to antimicrobial drugs, resistance to arsenic and production of toxins, have been attributed to some plasmids (Aslim *et al.*, 2002 and El-Hamshary *et al*., 2008). No relationship however could be established between the plasmid DNA of the isolates and polyethylene degradation in this study.

Biodegradation of the HDPE and LDPE films followed Norrish type I and II degradation as suggested by Sudhakar *et al*. (2007). This is evident by the various degradation products revealed by the FTIR spectra. It was not surprising to find the typical carbonyl band (1712 cm^{-1}) in the FTIR spectrum of treated films. The carbonyl band corresponds to the ketone and ester carbonyl groups and it is a typical product of oxidative degradation of PE (Gilan *et al*. 2004; Hadad *et al*., 2005). Unaged PE contains little or no carbonyl group. -OH stretching region of hydroxylic group 3100-3600 showed increment in the pretreated films. This is in agreement with the report of Guadagno *et al.* (2001) who observed an increase in the -OH stretching region of hydroxylic group 3050-3570, due to formation of hydroxyperoxide and alcohol during photo-oxidation. The main bands of the studied PE films consist of a band situated about 2900 cm^{-1} assignable to CH_2 as an asymmetric stretching, a band around 1461–1466 cm^{-1} revealing a bending deformation, and another band at 720–724 cm^{-1} which indicates a rocking deformation (Mouallif *et al*., 2011). Results showed a decrease in the carbonyl group after microbial treatment and removal of -OH bounded compounds (alcohol, hydroxyperoxide and carboxylic acids) in the 3100 – 3600 cm^{-1} region. These compounds which are degradation products along with the oxidised low molecular weight polymer have been reported to be assimilated by microorganisms (Hakkarainen & Albertsson, 2004). There was almost twice as much difference in the percentage reduction in carbonyl index in films treated with *Bacillus subtilis* (HDPE-10.5%;LDPE -26.7%) than with *Bacillus mycoides* (HDPE-21.1%;LDPE -13.7%). Likewise, similar difference was observed in the residual weight of microbial treated films.

Conclusion

The study revealed that initial abiotic treatment of the PE films ensured initiation of degradation. Abiotic pre-treatment lead to introduction of oxygen in the polymer matrix, to form oxygen containing compounds which were made available for utilization by the bacteria. *Bacillus mycoides* and *Bacillus subtilis* grew better on abiotically weathered PE films than on un-pretreated PE films. On the basis of this study it can be concluded that *Bacillus mycoides* and *Bacillus subtilis* indigenous to the Niger Delta mangrove soil have potential for use in biodegradation of PE.

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